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Protective effect of *l*-cis-diltiazem on hypercontracture of rat myocytes induced by veratridine

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Abstract

The protective effect of *l-cis*-diltiazem, the stereoisomer of *d-cis*-diltiazem, was studied against the veratridine-induced hypercontracture of rat myocytes. Veratridine increased both $[Na^+]_i$ and $[Ca^{2+}]_i$, but did not cause hypercontracture in the absence of extracellular Ca^{2+}. Both *l-cis*-diltiazem (0.1–10 μM) and *d-cis*-diltiazem (10–30 μM) inhibited the hypercontracture and the increase in $[Ca^{2+}]_i$ in a concentration-dependent manner. However, *l-cis*-diltiazem did not exert a negative inotropic effect in K^+ (20 mM)-depolarized rat papillary muscles even at a dose of 10 μM. As seen in the case of tetrodotoxin, *l-cis*-diltiazem and *d-cis*-diltiazem also suppressed the increase in $[Na^+]_i$. The results show that *l-cis*-diltiazem prevents the veratridine-induced hypercontracture of myocytes by suppression of the $[Ca^{2+}]_i$ increase. The attenuation of the $[Ca^{2+}]_i$ increase by *l-cis*-diltiazem was not dependent on inhibition of Ca^{2+} channels, but was partly due to inhibition of excessive Na^+ entry via veratridine-modified Na^+ channels.

Keywords: *l-cis*-Diltiazem; *d-cis*-Diltiazem; Veratridine; Ca^{2+} overload; Na^+ overload; Myocyte; (Rat)

1. Introduction

Ca^{2+} overload at reperfusion after ischemia (Bourdillon and Poole-Wilson, 1982; Jeremy et al., 1992) and reoxygenation after hypoxia (Crake and Poole-Wilson, 1986) is considered to be involved in the final stage of ischemia/reperfusion injury of myocytes (Nayler, 1981; Farber, 1982; Steenbergen et al., 1990). Recently, several reports have suggested that Na^+ overload during ischemia induces Ca^{2+} overload via reversal of the Na^+/Ca^{2+} exchanger (Murphy et al., 1988; Tani and Neely, 1989; Haigney et al., 1992; Silverman and Stern, 1994). Intracellular Na^+ accumulates during ischemia/reperfusion as a result of activation of the Na^+/H^+ exchanger to extrude accumulated H^+ (Karmazyn and Moffat, 1993; Nishida et al., 1993), depolarization, accumulation of lysophosphatidylcholine (Undrovinas et al., 1992), and activation of Na^+ channel (Takeo et al., 1989; Butwell et al., 1993; Silverman and Stern, 1994).

d-cis-Diltiazem, a benzothiazepine Ca^{2+} channel antagonist, exerts a protective effect against ischemia/reperfusion injury (Ngai et al., 1983; Lopaschuk et al., 1992), although it is thought that the Ca^{2+} channel antagonist is not effective on Ca^{2+} overload after reperfusion or reoxygenation (Watts et al., 1980). Recently, it was shown that *l-cis*-diltiazem, the stereoisomer of *d-cis*-diltiazem, possesses a protective effect against the ischemia/reperfusion injury in the rat working heart (Van Amsterdam et al., 1990; Nasa et al., 1990), although it is less potent as a Ca^{2+} channel antagonist than the *d*-isomer (Nagao et al., 1982). The blockade of L-type Ca^{2+} channels does not seem to contribute to the protective action of *l-cis*-diltiazem.

In the present study, we studied the direct effect of *l-cis*-diltiazem on morphological changes of rat isolated myocytes induced by veratridine. Veratridine inhibits the inactivation of the Na^+ channel and causes a continuous influx of Na ions into the depolarized cell (Honerjager, 1982). Furthermore, to investigate the possible mechanism of action of *l-cis*-diltiazem, we examined the change in intracellular concentration of Na^+ and Ca^{2+} in myocytes.

2. Materials and methods

2.1. Cell isolation

Male Sprague-Dawley rats (213–480 g) were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and

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artificially ventilated. The hearts were quickly excised and perfused according to the modified Langendorff method, as described by Powell et al. (1980). In brief, the hearts were perfused for 5 min with Krebs-Ringer (K-R) solution; the composition of Ca^{2+}-free K-R solution was 118.5 mM NaCl, 14.5 mM $NaHCO_3$, 2.6 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$ and 11.1 mM glucose; pH 7.4. Then the solution was switched to K-R solution with trypsin (100 U/ml, Funakoshi) and 25 μM $CaCl_2$. Following the 10-min perfusion, the hearts were perfused with K-R solution containing 0.1% collagenase (type I, Sigma) and 25 μM $CaCl_2$ until they became soft. All the solutions were aerated with 95% O_2/5% CO_2 and maintained at 37°C. The ventricle was dissected and minced in the K-R solution containing 0.1% bovine serum albumin (essentially fatty acid-free fraction V, Sigma), trypsin inhibitor (type III-0, Sigma) and 25 μM $CaCl_2$, and then incubated for 10 min at 37°C. The cell suspension was filtered through a 250-μm nylon mesh, centrifuged for 1 min and washed twice with centrifugation as above. Finally, the isolated cells were resuspended in K-R solution.

2.2. Evaluation of morphological changes of cardiac myocytes

Cells were lightly attached in a perfusion chamber and superfused (1 ml/min, room temperature) with K-R solution containing 1 mM $CaCl_2$, aerated with 95% O_2/5% CO_2. Cells were driven by an electrical stimulator (2 Hz, 10 ms). Veratridine (20 μM) was administered for 5 min in the K-R solution. Morphological changes of myocytes were examined under a microscope (IMT-2, Olympus, Tokyo, Japan). Each drug was perfused 20 min before and during the perfusion with veratridine. Under a microscope, viable and non-viable cells appear as rod-shaped and round-shaped, respectively. The number of cells that morphologically changed from a rod shape to a round shape after perfusion with veratridine was counted and expressed as a percentage of total cells.

2.3. Measurement of fluorescence ratio of fura-2 and SBFI in cardiac myocytes

For measurement of a change in $[Ca^{2+}]_i$ of cardiac myocytes, a Ca^{2+}-sensitive fluorescent dye, fura-2/acetoxymethylester (fura-2/AM), was used. Fura-2/AM (1 mM, dissolved in dimethylsulfoxide, DMSO) was prepared in K-R solution containing 100 μM $CaCl_2$ and 1% bovine serum albumin (fraction V, Sigma) and applied to isolated myocytes at a final concentration of 5 μM. After the cell suspension was incubated for 10 min at room temperature, myocytes were washed twice and suspended in K-R solution containing 1 mM $CaCl_2$. The fura-2-loaded cells were superfused (1 ml/min, room temperature) with K-R solution containing 1 mM $CaCl_2$, which was aerated with 95% O_2/5% CO_2, and were electrically stimulated (2 Hz, 10 ms). Then the myocytes were illuminated with a dual wavelength fluorometer (CAM-230, Japan Spectroscopic, Tokyo, Japan). Video images were digitized, using a silicon-intensified target camera and an Argus-50/Ca system (Hamamatsu Photonics). The fluorescence ratio was calculated from the value of the fluorescence intensity with 340-nm and 380-nm excitation. The protocol for drug perfusion was the same as that for the measurement of morphological changes. The digital images were obtained every 30 s from 1 min before the addition of veratridine to the end of the experiment.

Na^+-binding benzofuran isophthalate (SBFI/AM), a Na^+-sensitive fluorescent probe, was used for the measurement of changes in $[Na^+]_i$. SBFI/AM (1 mM, dissolved in DMSO) was mixed with Cremophor and prepared in the K-R solution containing 100 μM $CaCl_2$ and 1% bovine serum albumin. Isolated myocytes were loaded with SBFI/AM (10 μM) for 140 min at room temperature. The $[Na^+]_i$ measuring method and the protocol were the same as those for the $[Ca^{2+}]_i$ measurements.

2.4. Papillary muscle contraction

Male Sprague-Dawley rats (440–470 g) were anesthetized with sodium pentobarbital and left ventricular papillary muscles were isolated. The muscle was suspended in an organ bath filled with 10 ml K-R solution (37°C) containing 2.5 mM $CaCl_2$, which was gassed with 95% O_2/5% CO_2, and was driven by field stimulation (0.2 Hz, 5 ms). The isometric contraction was measured by a strain-gauge transducer. The solution in the organ bath was switched to K-R solution with 25 mM KCl, containing 2.5 mM $CaCl_2$, to depolarize the muscles, and then drugs were added cumulatively. The developed tension before the addition of drugs represented 100%.

2.5. Chemicals

l-cis-Diltiazem and *d-cis*-diltiazem were synthesized at Tanabe Seiyaku (Osaka, Japan). Veratridine was from Sigma (St. Louis, MO, USA) and dissolved in ethanol. The final concentration of ethanol was about 0.2%.

2.6. Statistical analysis

The results represent means ± S.E.M. of several experiments. Each experiment was done on myocytes isolated from different animals. The significance of differences was determined with an analysis of variance followed by Scheffe's method.

3. Results

3.1. Veratridine-induced morphological changes in myocytes and effects of drugs

Fig. 1 shows the effects of *l-cis*-diltiazem and *d-cis*-diltiazem on the number of non-viable cells after the treat-

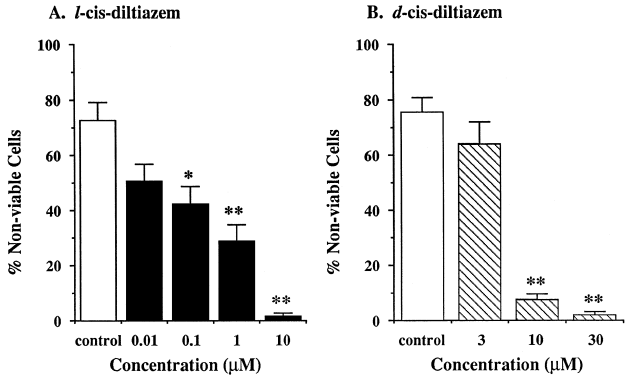

Fig. 1. Effects of *l-cis*- and *d-cis*-diltiazem on the viability of rat myocytes after the addition of veratridine (A, *l-cis*-diltiazem; B, *d-cis*-diltiazem). The number of round cells is expressed as a percentage of the number of rod-shaped cells (viable cells) before the addition of veratridine. Each column represents the mean ± S.E.M. of 4–9 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

ment with veratridine. About 70% of rod-shaped cells became round cells 5 min after the addition of veratridine (20 μM). The round cells did not return to their former rod shape upon repeated washing with fresh K-R solution. Since the round cells did not constrict upon electrical stimulation anymore, the irreversible change of the cell shape was assumed to represent the hypercontracture of myocytes. *l-cis*-Diltiazem inhibited the hypercontracture concentration dependently and 98% of the cells were rod-shaped at the concentration of 10 μM. *d-cis*-Diltiazem also inhibited the rounding of cells and the rod-shaped cells accounted for 92% and 98% of the total cells at the concentration of 10 μM and 30 μM, respectively. *d-cis*-Diltiazem was less potent than *l-cis*-diltiazem in protecting the myocytes. Tetrodotoxin (0.01 μM), a Na^+ channel blocker, also strongly prevented the rounding of cells (94.8 ± 1.4% inhibition, $n = 4$). Veratridine did not cause morphological changes of the cells in Ca^{2+}-free extracellular fluid (data not shown).

3.2. Effects of drugs on veratridine-induced changes in the fluorescence ratio of fura-2

In fura-2-loaded myocytes, the fluorescence ratio increased just after the administration of veratridine, and was followed by the morphological changes. The analysis of individual cells also demonstrated that the increase in fluorescence ratio occurred before rounding of the cells. At the concentration of 10 μM, *l-cis*-diltiazem and *d-cis*-diltiazem inhibited the increase in the fluorescence ratio by about 65% and 60%, respectively. Fig. 2A represents the time-course change of the fluorescence ratio in the absence and the presence of drugs. Veratridine increased $[Ca^{2+}]_i$ time dependently and the change in the ratio after 5 min was about 1.0. *l-cis*-Diltiazem and *d-cis*-diltiazem (10 μM) suppressed the change in the ratio to about 0.3 and 0.4, respectively. *l-cis*-Diltiazem inhibited the increases in the fluorescence ratio at 3–10 μM. Although statistically not significant, *l-cis*-diltiazem tended to suppress the increase in fluorescence ratio at 0.1 and 1.0 μM as well. *d-cis*-Diltiazem produced a significant inhibition at 10 μM (Fig. 2B). Tetrodotoxin (0.01 μM) suppressed the change in ratio to about 0.3 ($n = 2$). Veratridine did not increase the fluorescence ratio by removal of Na^+ from extracellular fluid (data not shown).

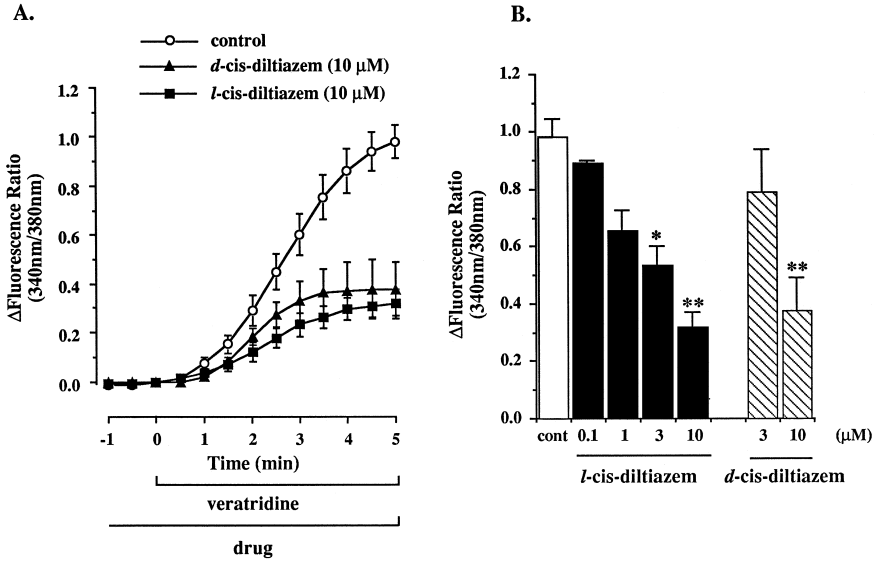

Fig. 2. Effects of *l-cis*-diltiazem and *d-cis*-diltiazem on veratridine-induced changes in the fluorescence ratio ($[Ca^{2+}]_i$ level) of fura-2-loaded rat myocytes (A: time dependency, B: concentration dependence 5 min after the addition of veratridine). Each point represents the mean ± S.E.M. of 3–11 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

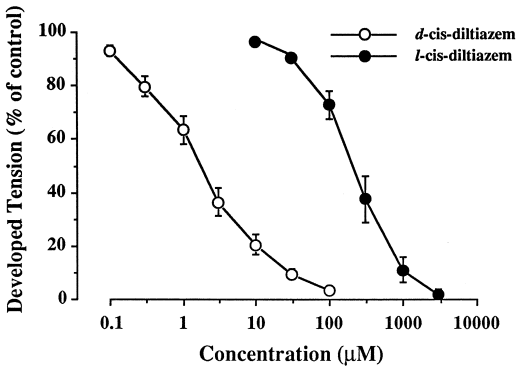

Fig. 3. Effects of *l-cis*-diltiazem and *d-cis*-diltiazem on contraction of rat left ventricular papillary muscles depolarized by 25 mM KCl. Each point represents the mean ± S.E.M. of 4 experiments.

3.3. Effects of drugs in K^+-depolarized rat papillary muscle

To determine the effects of *l-cis*-diltiazem and *d-cis*-diltiazem on L-type Ca^{2+} channels, the contraction was monitored in rat left ventricular papillary muscle depolarized by KCl. As shown in Fig. 3, *l-cis*-diltiazem concentration dependently suppressed the developed tension at concentrations over 30 μM and completely blocked it at 3 mM. *d-cis*-Diltiazem inhibited the contraction of depolarized muscles from 0.3 μM to 100 μM. Therefore, *l-cis*-diltiazem was about 100-times less potent than *d-cis*-diltiazem in blocking the cardiac L-type Ca^{2+} channels (pD_2, *l-cis*-diltiazem, 3.59 ± 0.12; *d-cis*-diltiazem, 5.73 ± 0.10). Tetrodotoxin (1 μM) did not influence the contraction of depolarized muscles (data not shown).

3.4. Effects of drugs on veratridine-induced changes in the fluorescence ratio of SBFI

Fig. 4 presents the effects of *l-cis*-diltiazem and *d-cis*-diltiazem on the change in fluorescence ratio of SBFI induced by veratridine (time-dependent (A) and drug concentration-dependent (B) changes in fluorescence ratio). The fluorescence ratio started to increase just after the perfusion of veratridine, and after 5 min the change in the ratio was about 0.05. *l-cis*-Diltiazem and *d-cis*-diltiazem inhibited the increases in the ratio at 10 μM by 45% and 40%, respectively. Tetrodotoxin inhibited the increase in the fluorescence ratio by 40% at 0.01 μM ($n = 4$) and by 100% at 3 μM ($n = 2$), respectively. The fluorescence ratio of SBFI increased to 0.1 even during the perfusion of Ca^{2+}-free K-R solution 5 min after the administration of veratridine; morphological changes did not occur (data not shown).

4. Discussion

4.1. Effect of veratridine

Veratridine binds to the open state of site-2 Na^+ channels and allows an excessive influx of Na ions into the cell (Khodorov, 1985). In this report, veratridine increased $[Na^+]_i$ and $[Ca^{2+}]_i$ of myocytes and then induced hypercontracture. When we analyzed the change in the fluorescence ratio of fura-2 in each cell, the increase in $[Ca^{2+}]_i$ was followed by hypercontracture of the cell. Under the extracellular Ca^{2+}-free condition, veratridine increased $[Na^+]_i$ without changing the shape of cells. Moreover,

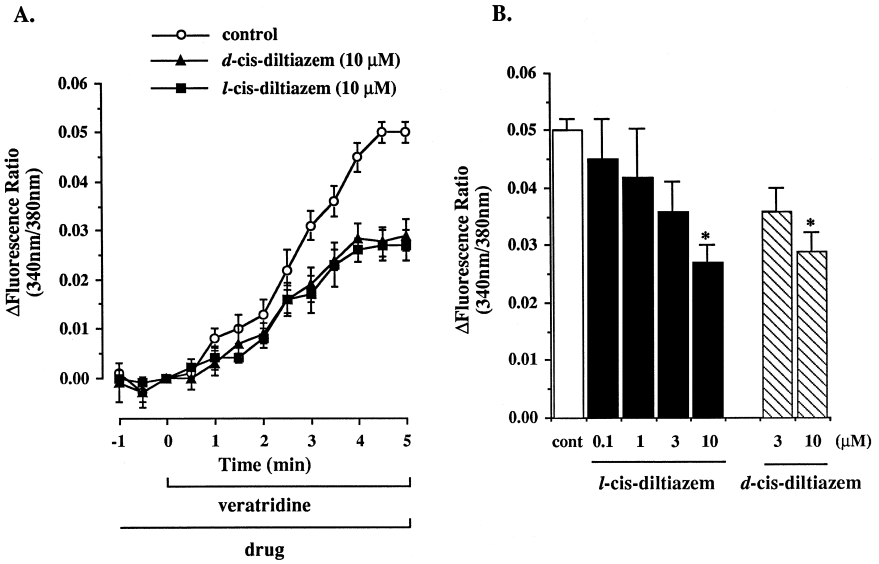

Fig. 4. Effects of *l-cis*-diltiazem and *d-cis*-diltiazem on the veratridine-induced change in fluorescence ratio ($[Na^+]_i$ level) of SBFI-loaded rat myocytes (A, time dependence; B, concentration dependence 5 min after the addition of veratridine). Each value represents the mean ± S.E.M. of 4–14 experiments. * $P < 0.05$ vs. control.

tetrodotoxin, a fast Na^+-channel blocker, inhibited both the increase in $[Na^+]_i$ and the hypercontracture of cells induced by veratridine. Thus, the veratridine-induced hypercontracture is mediated by Na^+ overload, which leads to Ca^{2+} overload.

Intracellular Na^+ accumulation has been reported to precede Ca^{2+} overload and subsequent damage of myocardium during anoxia, ischemia and reperfusion (Murphy et al., 1988; Tani and Neely, 1989, 1990). Elevated $[Na^+]_i$ may increase $[Ca^{2+}]_i$ via incorporation of extracellular Ca^{2+} by reversing or by stopping the extrusion of intracellular Ca^{2+} through the Na^+/Ca^{2+} exchanger. The excessive influx of Na^+ through the veratridine-modified Na^+ channel, thus, also induces Ca^{2+} overload via the Na^+/Ca^{2+} exchanger (Pauwels et al., 1989; Ver Donck and Borgers, 1991; Silverman and Stern, 1994). Therefore, the veratridine-induced hypercontracture could be considered as a model to simulate the damage of cardiac myocytes caused by accumulation of intracellular Ca^{2+} (Wermelskirchen et al., 1991; Hashizume et al., 1994).

4.2. Effects of l-cis-diltiazem and d-cis-diltiazem

Intracellular Ca^{2+} accumulation is considered to be the final stage leading to cell death in ischemia-reperfusion injury (Farber, 1982). Prevention of Ca^{2+} overload is expected to protect myocytes from cell death. In the present study, *l-cis*-diltiazem and *d-cis*-diltiazem suppressed both the hypercontracture and the increase in $[Ca^{2+}]_i$ induced by veratridine. Hence, the protective actions of both drugs against the veratridine-induced hypercontracture may be explained by their inhibitory action on Ca^{2+} overload. While 10 μM *l-cis*-diltiazem and *d-cis*-diltiazem exhibited 98% and 92% inhibition of the hypercontracture induced by veratridine, the inhibition of the $[Ca^{2+}]_i$ increase was only 65% and 60%, respectively. Similarly, tetrodotoxin (0.01 μM) inhibited almost completely the hypercontracture, but only partially the increase in $[Ca^{2+}]_i$ induced by veratridine. Therefore, the results suggest there is a threshold $[Ca^{2+}]_i$ level which causes hypercontracture of myocytes. There was a dissociation in the potency of *l-cis*-diltiazem to cause cell rounding and to increase $[Ca^{2+}]_i$. Nasa et al. (1990) reported that *l-cis*-diltiazem inhibited the accumulation of nonesterified fatty acid in the isolated working heart, but the correlation between this effect and intracellular Ca ion concentration was not clear. *l-cis*-Diltiazem is considered to prevent veratridine-induced cell damage through the mechanism described above, although details of its mechanism of action are not clear.

We investigated the effects of *l-cis*-diltiazem and *d-cis*-diltiazem on the rat K^+-depolarized papillary muscle to examine whether L-type Ca^{2+} channels contribute to the protective effects of these drugs. *l-cis*-Diltiazem was less potent than *d-cis*-diltiazem in blocking L-type Ca^{2+} channels. Furthermore, *l-cis*-diltiazem suppressed the hypercontracture at 10 μM, a concentration at which the tension of muscle was least affected. Therefore, these results indicate that the protective effect of *l-cis*-diltiazem is not mediated by the blockade of L-type Ca^{2+} channels. On the contrary, *d-cis*-diltiazem caused a 70% inhibition of the developed tension at 3 μM without affecting the hypercontracture. Therefore, the cardioprotective effect of *d-cis*-diltiazem at higher concentrations ($\geq$ 10 μM) also may be due to some mechanism other than the inhibition of Ca^{2+} channels. It has been shown that *d-cis*-diltiazem blocks Na^+ channels (Nakajima et al., 1975). In the present study, 10 μM *d-cis*-diltiazem inhibited the increase in $[Na^+]_i$ induced by veratridine. *l-cis*-Diltiazem, like *d-cis*-diltiazem, also suppressed the increase in $[Na^+]_i$ induced by veratridine. Although a relatively higher concentration of *l-cis*-diltiazem was needed to inhibit increases in $[Ca^{2+}]_i$ and $[Na^+]_i$ than to inhibit hypercontracture, tetrodotoxin (0.01 μM) also revealed different potencies for hypercontracture ($>$ 90% inhibition), Ca^{2+} movement (about 60% inhibition), and Na^+ movement (about 40% inhibition). Thus, *l-cis*-diltiazem may suppress veratridine-modified Na^+ channels, thereby inhibiting Ca^{2+} overload and hypercontracture. If the detrimental effect of intracellular Na^+ accumulation causes Ca^{2+} overload via the Na^+/Ca^{2+} exchanger (Murphy et al., 1988; Van Echteld et al., 1991), inhibition of the Na^+/Ca^{2+} exchanger would protect myocytes from damage. Thus, inhibition of the Na^+/Ca^{2+} exchanger might be the potential mechanism of action for the inhibitory effects of *l-cis*-diltiazem against Ca^{2+} overload.

In conclusion, *l-cis*-diltiazem inhibited the veratridine-induced hypercontracture of rat myocytes without blocking L-type Ca^{2+} channels. This effect may in part be due to the suppression of excessive Na^+ entry via veratridine-modified Na^+ channels, which causes intracellular Ca^{2+} overload.

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